(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 719 862 A2

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
03.07.1996 Bulletin 1996/27

(21) Application number: **95309454.7**

(22) Date of filing: **27.12.1995**

(51) Int Cl.[6]: **C12N 15/31**, C07K 14/195, C12N 15/74, C12N 1/21, C12N 9/78 // (C12N1/21, C12R1:01)

(84) Designated Contracting States:
CH DE FR GB LI

(30) Priority: **28.12.1994 JP 337652/94**

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(54) A regulatory factor for expression of nitrilase gene and a gene thereof

(57) The invention relates to a two component regulatory factor which activates a nitrilase gene promoter, comprising a polypeptide having the amino acid sequence of SEQ ID No: 1 and a polypeptide having the amino acid sequence of SEQ ID No: 2. Nitrilase can be produced by introducing the DNA coding for the regulatory factor together with a nitrilase gene containing a promoter region into a microorganism of the genus Rhodococcus.

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Printed by Jouve, 75001 PARIS (FR)

Description

FIELD OF THE INVENTION

The present invention relates to a regulatory factor involved in expression of a nitrilase gene and a DNA coding for the same and particularly to a regulatory factor derived from the strain Rhodococcus erythropolis SK92 and activating a nitrilase gene promoter, as well as to DNAs coding for the same, a recombinant plasmid containing the DNAs and a transformant transformed with said recombinant plasmid.

BACKGROUND OF THE INVENTION

As known processes of producing organic acids by conversion from their corresponding nitriles, mention may be made of chemical synthetic means and biological means. The latter involves the use of a microorganism or a microorganism-derived enzyme as a catalyst to hydrolyze nitriles, so this means is advantageous in that organic acids can be produced under mild conditions. Microorganisms belonging to the genus Rhodococcus are known as such catalysts for use in production of amides or organic acids by hydration or hydrolysis of their corresponding nitriles (see Japanese Laid-Open Patent Publication Nos. 251,192/1991, 91,189/1987, 470/1990, and 84,198/1990).

As compared with the above-mentioned conventional processes, the use of a nitrilase gene cloned for hydrolysis of nitriles by genetic recombination is expected to drastically improve the catalytic ability of the microorganism to hydrate nitriles because the microorganism can be engineered to contain multiple copies of the same gene. To obtain such a catalyst organism with higher catalytic activity, the present inventors successfully cloned a nitrilase gene from the strain Rhodococcus erythropolis SK92 and constructed a plasmid by inserting said gene into a region downstream of an E. coli lactose promoter. By introducing this plasmid into E. coli, the organism came to exhibit higher nitrilase activity during incubation in the presence of IPTG (isopropyl-β-D-thiogalactoside). The present inventors further attempted to obtain a transformant of the genus Rhodococcus to attain higher performance as a catalyst organism. In this attempt, the nitrilase gene was inserted into a Rhodococcus-E. coli hybrid plasmid vector (see Japanese Laid-Open Patent Publication Nos. 64,589/1993 and 68,566/1993), and the vector thus constructed was introduced into a microorganism of the genus Rhodococcus. However, no nitrilase activity was expressed, and there is demand for a method of permitting the expression of nitrilase activity in a transformant of the genus Rhodococcus.

SUMMARY OF THE INVENTION

The present inventors speculated that the gene derived from the genus Rhodococcus is not expressed because the promoter for the nitrilase gene fails to function, and that a gene coding for a regulatory factor that allows the promoter to function might be present somewhere on the chromosomal DNA derived from SK92. Through screening, the present inventors found it in a region upstream of the nitrilase structural gene and succeeded thereby in expression of nitrilase activity in a transformant of the genus Rhodococcus.

That is, the present invention relates to a regulatory factor consisting of 2 components i.e. a polypeptide having the amino acid sequence of SEQ ID No: 1 and a polypeptide having the amino acid sequence of SEQ ID No: 2 to activate the nitrilase gene promoter, as well as to DNAs coding for them.

Introduction of the gene coding for the regulatory factor of the invention along with the nitrilase gene containing its promoter permits a microorganism of the genus Rhodococcus to produce nitrilase.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a schematic drawing of deletion plasmids, where the arrows on the DNA fragment from SK92 indicate the location and direction of the gene coding for the regulatory factor of the invention and the gene coding for nitrilase, respectively.

Fig. 2 shows a restriction enzyme map of recombinant plasmid pSK108.

DETAILED DESCRIPTION OF THE INVENTION

Hereinafter, the present invention is described in detail. The present invention is practiced in the following steps.

(1) Preparation of chromosomal DNA from the strain SK92:
Chromosomal DNA is isolated from Rhodococcus erythropolis SK92.

(2) Construction of a DNA Library:
The chromosomal DNA is cleaved with restriction enzymes, and a DNA fragment containing the target gene

is detected by Southern hybridization using the nitrilase gene of SK92 as probe. This fragment is inserted into a hybrid plasmid vector capable of replicating in cells of E. coli and the genus Rhodococcus to prepare a library.

(3) Transformation of E. coli and selection of recombinant DNA:

The recombinant library constructed in step (2) is used to prepare transformants. They are subjected to colony hybridization using the probe obtained in step (2) to select a colony carrying the target recombinant DNA.

(4) Preparation of recombinant plasmid:

A plasmid is prepared from the recombinant obtained in step (3).

(5) Transformation of a microorganism of the genus Rhodococcus and the nitrilase activity of the transformant:

The resulting plasmid is introduced into a microorganism of the genus Rhodococcus, and its nitrilase activity is determined.

(6) Deletion plasmids and nitrilase activity:

Deletion plasmids are prepared by deleting various regions from the plasmid obtained in step (4) to identify the region essential for expression of the nitrilase structural gene. The plasmids prepared are not necessary to be capable of replicating in E. coli and are sufficient if they include a DNA region capable of replicating in cells of the genus Rhodococcus.

(7) Nucleotide sequencing:

The nucleotide sequence of the region identified in step (6) is determined.

As the above hybrid plasmid vector, mention may be made of pK1, pK2, pK3 and pK4. These plasmids were introduced into R. rhodochrous ATCC 12674 and have been deposited respectively as R. rhodochrous ATCC 12674/pK1 (FERM BP-3728), R. rhodochrous ATCC 12674/pK2 (FERM BP-3729), R. rhodochrous ATCC 12674/pK3 (FERM BP-3730) and R. rhodochrous ATCC 12674/pK4 (FERM BP-3731) with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (see Japanese Laid-Open Patent Publication No. 68,556/1993).

As the above DNA region capable of replicating in cells of the genus Rhodococcus, mention may be made of those derived from plasmids pRC001, pRC002, pRC003 and pRC004, and these may be the whole of the plasmid or a partial fragment thereof. The above plasmids are derived respectively from the strains R. rhodochrous ATCC 4276, ATCC 14349, ATCC 14348 and IFO 3338 (see Japanese Laid-Open Patent Publication No. 68,556/1993).

Rhodococcus erythropolis SK92 has been deposited as FERM BP-3324 with the Fermentation Research Institute, Agency of Industrial Science and Technology. Plasmid pSK108 containing the nitrilase gene and the regulatory gene has been deposited as transformant JM109/pSK108 (FERM BP-5322) carrying said plasmid pSK108, with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology. The strain SK92 was previously identified as belonging to the genus Rhodococcus on the basis of its bacterial properties (see Japanese Laid-Open Patent Publication No. 280,889/1991). This organism is further identified as Rhodococcus erythropolis on the basis of the following detailed properties:

ITEMS EXAMINED	RESULTS
decomposition of adenine	+
decomposition of tyrosine	+
decomposition of urea	+
utilization	
inositol	+
maltose	−
mannitol	+
rhamnose	−
sorbitol	+
sodium m-hydroxy-benzoate	−
sodium benzoate	+
sodium citrate	+
sodium lactate	+
testosterone	+
acetamide	+
sodium pyruvate	+
growth in the presence of 0.02 % sodium azide	+
growth at 10 ℃	+
growth at 40 ℃	−
growth in the presence of 0.001 % crystal violet	−
growth in the presence of 0.3 % phenyl ethanol	−
growth in the presence of 5 % NaCl	+
growth in the presence of 7 % NaCl	+

EXAMPLES

Hereinafter, the present invention will be illustrated in detail by reference to the following examples which however are not intended to limit the scope of the invention.

Cloning of the nitrilase gene from SK92 and the expression thereof in E. coli and Rhodococcus will be further

illustrated in Reference Example.

(1) Preparation of chromosomal DNA from SK92

The strain SK92 was incubated at 30 °C for 72 hours under shaking in 100 ml MY medium (0.5 % polypeptone, 0.3 % Bacto-yeast extract, 0.3 % Bacto-molt extract). The cells were harvested and the pellet was suspended in 4 ml Saline-EDTA solution (0.1 M EDTA, 0.15 M NaCl, pH 8.0). 8 mg of lysozyme was added to the suspension. The suspension was incubated at 37 °C for 1 to 2 hours under shaking and then frozen. Then, 10 ml of Tris-SDS solution (1 % SDS, 0.1 M NaCl, 0.1 M Tris, pH 9.0) was added to it under gentle shaking, followed by addition of proteinase K (Merk) at a final concentration of 0.1 mg. The mixture was incubated under shaking at 37 °C for 1 hour and then at 60 °C . An equal amount of phenol saturated with TE (TE: 10 mM Tris, 1 mM EDTA, pH 8.0) was added to the mixture, stirred, and centrifuged. A 2-fold excess amount of ethanol was added to the upper layer, and the DNA was recovered using a glass rod. The phenol was removed successively with 90 %, 80 % and 70 % ethanol. Then, the DNA was dissolved in 3 ml TE buffer, and a solution of ribonuclease A (previously treated by heating at 100 °C for 15 min.) was added to it in an amount of 10 μ g/ml. The mixture was incubated at 37 °C for 30 minutes under shaking, followed by addition of proteinase K. The mixture was incubated at 37 °C for 30 minutes under shaking. An equal amount of TE-saturated phenol was added to the mixture, and it was separated by centrifugation into upper and lower layers. The upper layer was subjected twice to the same procedure, followed by the same procedure of extraction with an equal amount of chloroform containing 4 % isoamyl alcohol (these procedures are referred to hereinafter as phenol treatment). Then, a 2-fold excess amount of ethanol was added to the upper layer and the DNA was recovered with a glass rod whereby the chromosomal DNA was obtained.

(2) Construction of a DNA library

10 μ l plasmid pSK002 prepared by inserting into vector pUC118 a DNA fragment containing the nitrilase gene from the strain SK92 (see Reference Example) was cleaved at 37 °C for 2 hours with a mixture of 2 μ l of restriction enzyme Sac I, 10μ l of the reaction buffer (10-fold conc.), and 78 μ l of sterilized water, and the digest was electrophoresed on 0.7 % agarose gel to separate an Sal I fragment, 1.1 kb long.

Separately, the chromosomal DNA from SK92 obtained in step (1) was digested with Eco RI, electrophoresed on agarose gel and subjected to Southern hybridization where the above 1.1 kb Sac I fragment, labeled using a DIG DNA Labeling Kit (Boehringer Mannheim), was used as the probe (Southern E.M., Mol. Bionl. 98, 503 (1975)) to detect an about 14 kb DNA fragment. A DNA fraction containing the 14 kb fragment hybridized with the probe was cut off from the agarose gel and then inserted into a separately prepared Eco RI-cleaved hybrid plasmid vector pK4 (FERM BP-3731 containing plasmid pRC004 from the genus Rhodococcus and vector pHSG299 from E. coli (see Japanese Laid-Open Patent Publication Nos. 64,589/1993 and 68,566/1993)).

The above pK4 fragment used as vector was prepared as follows: 10 μ l of the reaction buffer (10-fold conc.), 77 μ l of sterilized water and 2 μ l of restriction enzyme Eco RI were added to 10 μ l of vector pK4. The mixture was allowed to react at 37 °C for 2 hours, then treated with phenol, precipitated with ethanol, dried, and dissolved in 50 μ l sterilized water. 1 μ l of alkaline phosphatase (Takara Shuzo Co., Ltd.), 10 μ l of the reaction buffer (10-fold conc.) and 39 μ l of sterilized water were added to it. The mixture was allowed to react at 65 °C, treated with phenol, precipitated with ethanol, dried, and dissolved in sterilized water.

As described above, 1 μ l of the above DNA fraction containing the 14 kb fragment was inserted into the above Eco RI-cleaved pK4 by overnight reaction at 4°C using a ligation kit (Takara Shuzo Co., Ltd.) to prepare a DNA library.

(3) Transformation of E. coli and selection of recombinant DNA

E. coli JM109 (available from Takara Shuzo Co., Ltd.) was inoculated into 1 ml of LB medium (1 % Bacto-trypton extract, 0.5 % Bacto-yeast extract, 0.5 % NaCl) and pre-incubated at 37 °C for 5 hours. 100 μ l of the culture was inoculated into 50 ml of SOB medium (2 % Bacto-trypton, 0.5 % Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 1 mM MgSO4, 1 mM MgCl2) and incubated at 18 °C for 20 hours. The cells were recovered by centrifugation, and the pellet was suspended in 13 ml cold TF solution (20 mM PIPES-KOH, pH 6.0, 200 mM KCl, 10 mM $CaCl_2$, 40 mM $MnCl_2$), allowed to stand at 0 °C for 10 minutes and centrifuged again. After the supernatant was removed, the E. coli pellet was suspended in 3.2 ml of cold TF solution, followed by addition of 0.22 ml dimethyl sulfoxide. The suspension was allowed to stand at 0 °C for 10 minutes. 10 μ l of the recombinant plasmid (DNA library) prepared in step (2) was added to 200 μ l of the competent cells thus prepared. The mixture was incubated at 0 °C for 30 minutes, then heat-shocked at 42 °C for 30 seconds and cooled at 0 °C for 2 minutes, followed by addition of 0.8 ml of SOC medium (2 % Bacto-trypton, 0.5 % Bacto-yeast extract, 20 mM glucose, 10 mM NaCl, 2.5 mM KCl, 1 mM $MgSO_4$, 1 mM $MgCl_2$). The mixture was incubated at 37 °C for 60 minutes under shaking. The culture was plated in an amount of 200 μ l per plate on LB agar medium containing 100 μ g/ml ampicillin. The plate was incubated at 37 °C . Selection of transformants carrying the nitrilase gene from the colonies grown on the plate was carried out by colony hybridization in the following manner. The colonies grown on the plate were transferred to a nylon membrane (Biodyne A produced by Nippon Paul) and the microorganisms were lysed. The DNA

was fixed on the membrane and then hybridized with the probe (1.1 kb fragment) constructed in step (2), and the colony containing the target recombinant DNA was selected using a DIG Luminescent Detection Kit (Boehringer Mannheim).

(4) Preparation of recombinant plasmid

The transformant selected in step (3) was incubated at 37 °C overnight in 100 ml of LB medium, and the cells were harvested and washed with sterilized water. 5 ml of solution I (2 mM glucose, 10 mM EDTA, 25mM Tris-HCl buffer, pH 8.0) and 25 mg lysozyme were added to the cells. It was allowed to stand at 0 °C for 30 minutes. 10 ml of solution II (1 N NaOH, 5 % SDS) was added thereto, and the mixture was allowed to stand at 0 °C for 5 minutes. 7.5 ml of solution III (3 M sodium acetate, pH 4.8) was added thereto, and the mixture was allowed to stand at 0 °C for 30 minutes and centrifuged. 50 ml ethanol was added to the supernatant. It was centrifuged again to remove the supernatant. 5 ml of solution IV (10 mM sodium acetate, 50 mM Tris-HCl buffer, pH 8.0) and 2.5 μ l of 10 mg/ml ribonuclease A were added thereto. The mixture was allowed to stand at room temperature for 20 minutes, followed by addition of 12 ml ethanol. It was centrifuged, dried, and dissolved in sterilized water.

(5) Transformation of a microorganism of the genus Rhodococcus, and the nitrilase activity of the transformant

Rhodococcus rhodochrous ATCC 12674 at the logarithmic growth phase was harvested by centrifugation, washed 3 times with ice-cold sterilized water and suspended in sterilized water. 1 μg of plasmid pSK104 obtained in step (4) was mixed with 10 μ l of the cell suspension, and the mixture was cooled on ice. This mixture of the DNA and the microorganism was introduced into the chamber in a electroporation apparatus CET-200 (Japan Spectroscopic Co., Ltd.), and the sample was pulsed 20 times with a density of electric field of 3.8 kV/cm and a pulse width of 1 ms. The cell suspension thus treated was placed on ice for 10 minutes and heat-shocked at 37 °C for 10 minutes. 500 μ l of MYK medium (0.5 % polypeptone, 0.3 % Bacto-molt extract, 0.3 % Bacto-yeast extract, 0.2 % KH_2PO_4, 0.2 % K_2HPO_4 (pH 7.0)) was added thereto. The cell suspension was then incubated at 26 °C for 3 hours under shaking. The suspension was plated on an MYK agar plate containing 75 μg/ml kanamycin and incubated at 26 °C for 3 days.

The resultant transformant of the genus Rhodococcus was inoculated into 10 ml MYK medium containing 50 μ g/ml kanamycin and pre-incubated at 30 °C for 24 hours. 1 ml of the culture was added to 100 ml of GGP medium (1.5 % glucose, 0.1 % Bacto-yeast extract, 1.0 % sodium glutamate, 0.05 % KH_2PO_4, 0.05 % K_2HPO_4, 0.05 % $MgSO_4\ 7H_2O$ (pH 7.2)) containing 1.5 % ethylene cyanohydrin (ECH) as inducer and 75 μ g/ml kanamycin. The microorganism was incubated at 30 °C for 48 hours and harvested, and the pellet was suspended in 50 mM phosphate buffer, pH 7.7, and a part of the suspension was allowed to react at 30 °C for 20 minutes in 50 mM phosphate buffer, pH 7.7, containing 100 mM acrylonitrile. The reaction was stopped by addition of 1 N HCl, and the amount of acrylic acid formed in the reaction solution was determined by high performance liquid chromatography (HPLC). The result indicated the formation of 8 mM acrylic acid in the transformant ATCC 12674/pSK104. It was revealed that the gene coding for the regulatory factor necessary for expression of nitrilase is present upstream or downstream of the structural gene of nitrilase.

(6) Deletion plasmids and nitrilase activity

Because pSK104 was estimated to still contain a number of regions not required for expressing nitrilase, various deletion plasmids were prepared therefrom. Microorganisms transformed with the deletion plasmids were examined for their nitrilase activity (Table 1, Fig. 1).

Table 1.

Deletion plasmids and formation of acrylic acid		
	amount of formed acrylic acid (mM)	
	inducer (ECH)	
	absent	present
1) pSK102	0	0
2) pSK104	0.77	8.00
3) pSK105	0	1.71
4) pSK123	0	0
5) pSK124	0	0
6) pSK106	1.14	6.38
7) pSK107	0	3.40
8) pSK125	0	0
9) pSK126	0	0

Table 1. (continued)

Deletion plasmids and formation of acrylic acid		
	amount of formed acrylic acid (mM)	
	inducer (ECH)	
	absent	present
10) pSK127	0	0
11) pSK109	0	0
12) pSK108	0	8.05

As is evident from the table, ATCC12674/pSK108 (6.2 kb HindIII-EcoRV fragment) (FIG. 2) is of high nitrilase activity.

Additional deletion plasmids were constructed and examined for the gene coding for the regulatory factor. The result revealed that the gene is located within a far upstream region (about 3 kb BamHI-EcoRV fragment) from the structural gene of nitrilase.

(7) Nucleotide sequencing

The gene coding for the regulatory factor essential for expression of nitrilase, revealed in step (6), was sequenced using Fluorescence Sequencer ALFII (Pharmacia). The sequence analysis revealed the nucleotide sequence of SEQ ID No: 5, and the presence of 2 open reading frames coding respectively for the amino acid sequences of SEQ ID Nos: 1 and 2 was found. Comparison with Amino Acid Sequence Data Base NBRF (National Biomedical Research Foundation) suggested that the regulatory factor belongs to a family of two-component regulator. The nucleotide sequences of these open reading frames are shown in SEQ ID Nos: 3 and 4.

Reference Example

(1) Preparation of the chromosomal DNA from the strain SK92

The chromosomal DNA from SK92 was prepared in the same manner as in Example, step (1).

(2) Preparation of a probe and construction of a DNA library

Polymerase chain reaction was carried out using 100 μl solution containing 10 μl of DNA as substrate (diluted 20-fold), 10 μl of the reaction buffer (10-fold conc.), 4 μl of 5 mM dNTP, 5 μl (500 pmol) each of 5'-AACTGCTGGGA (AG)CACTTCCA-3' as primer #1 (20 nucleotides corresponding to the amino acid sequence NCWEHFQ) and 5'-GA(AG)TA(AG) TG(AG)CC(CG)AC(ACTG)GG(AG)TC-3' as primer #2 (20 nucleotides corresponding to the amino acid sequence DPVGHYS), and 1 μl of Tth DNA polymerase (Toyo Boseki). The above 2 primers were prepared on the basis of amino acid sequences having high homologies with known various nitrilases. The reaction involved 50 cycles each consisting of the incubation of the sample at 93 °C for 30 seconds (denaturation step), 45 °C for 30 seconds (annealing step) and 72 °C for 2 minutes (elongation step). A 410 bp DNA fragment coding for the nitrilase from SK92 was obtained from the reaction solution. This DNA fragment was labeled as probe using a DIG DNA Labeling Kit (Boehringer Mannheim).

10 μl of the reaction buffer (10-fold conc.), 37 μl of sterilized water and 3μl of restriction enzyme Sal I were added to 50 μl of the chromosomal DNA from SK92. The mixture was allowed to react at 37 °C for 2 hours, then precipitated with ethanol and electrophoresed on agarose gel. A DNA fragment, about 1.1 kb, was recovered using DNA PREP (DIA-IATRON). The DNA fragment was inserted into the Sal I site of E. coli vector pUC118 using a ligation kit (Takara Shuzo Co., Ltd.) whereby a recombinant DNA library was prepared.

The above pUC118 fragment was prepared in the following manner. 10 μl of the reaction buffer (10-fold conc.), 77 μl of sterilized water and 2 μl of restriction enzyme Sal I were added to 10 μl of pUC118. The mixture was allowed to react at 37 °C for 2 hours, then treated with phenol, precipitated with ethanol, dried, and dissolved in 50 μl of sterilized water. 1 μl of alkaline phosphatase (Takara Shuzo Co., Ltd.), 10 μl of the reaction buffer (10-fold conc.) and 39 μl of sterilized water were added thereto. The sample solution was allowed to react at 65 °C, treated with phenol, precipitated with ethanol, dried, and dissolved in sterilized water.

(3) Transformation of E. coli and selection of recombinant DNA

Competent cells of E. coli JM109 were prepared in the same manner as in Example, step (3). 10 μl solution (DNA library) containing the recombinant plasmid prepared in step (2) was added to 200 μl of the competent cells. The cells were allowed to stand at 0 °C for 30 minutes, then heat-shocked at 42 °C for 30 seconds and cooled at 0 °C for 2 minutes. 0.8 ml of SOC medium was added thereto, and the cells were incubated at 37 °C for 60 minutes under shaking. The culture was plated in an amount of 200 μl per plate onto LB agar medium containing 100 μg/ml ampicillin, followed by incubation at 37 °C. Selection of a transformant carrying the nitrilase gene from the

colonies grown on the agar medium was carried out by colony hybridization in the following manner. The transformants grown on the agar medium were transferred to a nylon membrane (Biodaine A produced by Paul Co., Ltd.) and they were lysed to fix DNA. The DNA was treated with the probe (410 bp fragment) prepared in step (2), and the colony containing the target recombinant DNA was selected using a DIG Luminescent Detection Kit (Boehringer Mannheim).

(4) Construction of recombinant plasmids and preparation of a restriction enzyme map

The transformant selected in step (3) was treated in the same manner as in Example, step (4). The recombinant plasmid pSK002 thus obtained was cleaved with several restriction enzymes to prepare a restriction enzyme map.

(5) Production of nitrilase by transformed E. coli and conversion of a nitrile into an acid

The JM109/pSK002 strain was inoculated into 1 ml of 2×YT medium (1.6 % Bacto-trypton, 1.0 % Bacto-yeast extract, 0.5 % NaCl) containing 50 μ g/ml ampicillin and incubated at 37 °C for 8 hours. 1 ml of the culture was inoculated into 100 ml of 2×YT medium containing 50 μ g/ml ampicillin and 1 mM IPTG, followed by incubation at 37 °C for 14 hours. After harvested, the microorganisms were suspended in 50 mM phosphate buffer, pH 7.7, and a part of the suspension was allowed to react at 30 °C for 20 minutes in 50 mM phosphate buffer, pH 7.7, containing 100 mM acrylonitrile. The reaction was stopped by addition of 1 N HCl, and the amount of acrylic acid formed in the reaction solution was determined by HPLC. In the control test, the strain JM109 before transformation was used. The result indicates that while no acrylic acid was detected in the host JM109, the formation of 18 mM acrylic acid was found in the transformant JM109/pSK002.

(6) Introduction of the DNA fragment containing the nitrilase gene into a hybrid plasmid vector

A DNA fragment (5.8 kb BglII-HindIII fragment) containing the nitrilase structual gene and a region speculated to contain its promoter were cloned into hybrid plasmid vector pK4 whereby plasmid pSK 120 was constructed.

(7) Transformation of a microorganism of the genus Rhodococcus and the nitrilase activity of the transformant

Rhodococcus rhodochrous ATCC 12674 at the logarithmic growth phase was harvested by centrifugation, washed 3 times with ice-cold sterilized water, and suspended in sterilized water. 10μ g cell suspension was mixed with 1 μ g of plasmid pSK120 obtained in step (6), and the mixture was then cooled on ice. This mixture of the DNA and the microorganism was introduced into the chamber in a gene-introducing unit CET-200 (Nippon Bunko) where the sample was pulsed 20 times with a density of electric field of 3.8 kV/cm and a pulse width of 1ms.

The cell suspension thus treated was placed on ice for 10 minutes and heat-shocked at 37 °C for 10 minutes. 500 μ l of MYK medium was added to the suspension and the mixture was then incubated at 26 °C for 3 hours under shaking. The culture was plated onto MYK agar medium containing 75 μg/ml kanamycin and incubated at 26 °C for 3 days.

The thus obtained transformant of the genus Rhodococcus was inoculated into 10 ml MYK medium containing 50 μ g/ml kanamycin and pre-incubated at 30 °C for 24 hours. 1 ml of the culture was added to 100 ml of GGP medium containing 75 μ g/ml kanamycin. 1.5 % ECH was added thereto as inducer. The transformant was incubated at 30 °C for 48 hours. After recovered, the cells were suspended in 50 mM phosphate buffer, pH 7.7, and their nitrilase activity was examined in the same manner as in step (5). No activity was found in it.

A number of references are cited herein, the disclosures of which are incorporated in their entireties by reference herein.

SEQUENCE LISTING

SEQ ID No: 1
LENGTH: 244
TYPE: amino acid
TOPOLOGY: linear
MOLECULAR TYPE: protein
ORIGINAL SOURCE
ORGANISM: Rhodococcus erythropolis
STRAIN: SK92
SEQUENCE:

15

MetAlaGlyAlaAspValHisAlaGlnGlyGlyThrAsnArgArg

30

AlaArgIleLeuValValAspAspGluLysHisValArgThrMet

45

ValThrTrpGlnLeuGluSerGluAsnPheAspValValAlaAla

60

AlaAspGlyAspAlaAlaLeuArgGlnValThrGluSerAlaPro

75

AspLeuMetValLeuAspLeuSerLeuProGlyLysGlyGlyLeu

90

GluValLeuAlaThrValArgArgThrAspAlaLeuProIleVal

105

ValLeuThrAlaArgArgAspGluThrGluArgIleValAlaLeu

120

AspLeuGlyAlaAspAspTyrValIleLysProPheSerProArg

135

GluLeuAlaAlaArgIleArgAlaValLeuArgArgThrThrAla

150

GluProProIisGluAlaAlaValGlnArgPheGlyAspLeuGlu

165

IleAspThrAlaAlaArgGluValArgLeuIisGlyIleProLeu

180

GluPheThrThrLysGluPheAspLeuLeuAlaTyrMetAlaAla

195

SerProMetGlnValPheSerArgArgArgLeuLeuLeuGluVal

210

TrpArgSerSerProAspTrpGlnGlnAspAlaThrValThrGlu

225

HisValHisArgIleArgArgLysIleGluGluAspProThrLys

240

ProThrIleLeuGlnThrValArgGlyAlaGlyTyrArgPheAsp

244

GlyGluArgAla

SEQ ID No: 2
LENGTH: 534
TYPE: amino acid
TOPOLOGY: linear
MOLECULAR TYPE: protein
ORIGINAL SOURCE
ORGANISM: Rhodococcus erythropolis
STRAIN: SK92
SEQUENCE:

15

MetMetThrAspThrLeuProSerSerSerArgTrpThrLeuGlu

30

GlyProHisLeuGlnProLeuGlnGlyGluAlaLeuAlaAspLeu
45

HisAlaArgThrLeuGluMetIleThrSerGlyArgGluLeuHis
60

GluThrLeuGluValValAlaArgGlyIleGluGluLeuMetPro
75

GlyLysArgCysAlaIleLeuLeuLeuAspAsnThrGlyProVal
90

LeuArgCysGlyAlaAlaProThrMetSerAlaProTrpArgArg
105

TrpIleAspSerLeuValProGlyProMetSerGlyGlyCysGly
120

ThrAlaValHisLeuGlyGluProValIleSerTyrAspValAla
135

AspAspProLysPheArgGlyProPheArgAlaAlaAlaLeuHis
150

GluGlyIleArgAlaCysTrpSerThrProValThrSerGlyAsp
165

GlyThrIleLeuGlyThrPheAlaIleTyrGlySerValProAla
180

PheProAlaGlnGlnAspValAlaLeuValThrGlnCysThrAsp
195

LeuThrAlaAlaValIleThrThrHisLysLeuHisGlnAspLeu
210

SerMetSerGluGluArgPheArgArgAlaPheAspSerAsnVal
225

ValGlyMetAlaLeuLeuAspGluSerGlySerSerIleArgVal
240

AsnAspThrLeuCysAlaLeuThrAlaAlaProProArgArgLeu

255

LeuGlyHisProMetGlnGluIleLeuThrAlaAspSerArgGlu

270

ProPheAlaAsnGlnLeuSerSerIleArgGluGlyLeuThrAsp

285

GlyGlyGlnLeuAspGlyArgIleGlnThrThrGlyGlyArgTrp

300

IleProValHisLeuSerIleSerGlyMetTrpThrThrGluArg

315

GluPheMetGlyPheSerValHisValLeuAspIleSerGluArg

330

LeuAlaAlaGluArgAlaArgGluGluGlnLeuGluAlaGluVal

345

AlaArgHisThrAlaGluGluAlaSerArgAlaLysSerThrPhe

360

LeuSerGlyMetThrHisGluValGlnThrProMetAlaValIle

375

ValGlyPheSerGluLeuLeuGluThrLeuAspLeuAspGluGlu

390

ArgArgGlnCysAlaTyrArgLysIleGlyGluAlaAlaLysHis

405

ValIleSerLeuValAspAspValLeuAspIleAlaLysIleGlu

420

AlaGlyAlaIleThrLeuGlnAspGluAspIleAspLeuSerGlu

435

GluValAlaThrIleValGluMetLeuGluProIleAlaArgAsp

450

ArgAspArgAspValCysLeuArgTyrValProProGlnThrPro

465

ValHisValCysSerAspArgArgArgValArgGluValLeuLeu

480

AsnIleValSerAsnGlyIleLysTyrAsnArgLeuGlyGlyVal

495

ValAspProProThrGlySerGlyAlaAlaArgProArgGlnThr

510

ArgAlaProAspTyrProAlaThrProThrThrAsnSerSerSer

525

ProSerThrGlyTrpGluSerArgProArgGlyCysLysGlyArg

534

GlySerValLeuArgSerProAlaArg

SEQ ID No: 3
LENGTH: 735
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
ORIGINAL SOURCE
ORGANISM: Rhodococcus erythropolis
STRAIN: SK92
SEQUENCE:

ATG GCC GGA GCG GAC GTC CAC GCC CAG GGT GGC ACG AAT CGA CGT 45

GCA CGC ATC CTC GTC GTC GAC GAC GAA AAA CAC GTG CGC ACG ATG 90

GTG ACG TGG CAA CTC GAA TCG GAG AAT TTC GAT GTT GTC GCT GCG 135

GCA GAC GGA GAT GCG GCA CTG CGT CAG GTC ACT GAG AGC GCA CCC 180

GAT TTG ATG GTG CTC GAT CTG TCG CTC CCG GGG AAA GGT GGG TTG 225

GAA GTG CTC GCT ACG GTC CGC AGA ACC GAT GCA CTG CCT ATC GTC 270

GTG CTC ACA GCA CGC CGC GAT GAA ACC GAA CGG ATC GTC GCG CTG 315

GAT CTC GGC GCC GAT GAC TAC GTC ATC AAA CCG TTC TCC CCG CGG 360

GAA TTG GCC GCC CGT ATC CGG GCA GTG CTT CGT CGA ACC ACA GCT 405

GAA CCC CCA CAC GAG GCG GCG GTT CAG CGA TTC GGT GAC CTA GAG 450

ATC GAC ACC GCT GCG CGC GAG GTT CGG CTC CAC GGG ATA CCG CTC 495

GAG TTC ACC ACC AAG GAG TTC GAT CTG CTG GCC TAT ATG GCC GCA 540

TCA CCG ATG CAG GTC TTC AGC CGA CGC AGA TTG TTG CTC GAG GTG 585

TGG CGA TCG TCG CCC GAC TGG CAG CAG GAC GCC ACC GTG ACC GAG 630

CAC GTG CAC CGC ATT CGC CGC AAG ATC GAA GAA GAT CCC ACC AAA 675

CCG ACG ATC CTG CAG ACA GTG CGG GGA GCC GGT TAC CGT TTC GAC 720

GGA GAG CGT GCA TGA 735

SEQ ID No: 4
LENGTH: 1605
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear

ORIGINAL SOURCE
ORGANISM: Rhodococcus erythropolis
STRAIN: SK92
SEQUENCE:

ATG ATG ACC GAC ACA CTG CCC TCC TCG TCC CGT TGG ACC CTT GAA 45

GGC CCG CAT CTC CAG CCG CTG CAG GGT GAG GCC CTG GCG GAT CTC 90

CAC GCC CGT ACG CTC GAG ATG ATC ACT TCC GGG AGA GAA TTG CAC 135

GAG ACA CTC GAG GTG GTC GCC CGC GGC ATC GAG GAA CTG ATG CCG 180

GGC AAA CGT TGC GCA ATT CTG TTG CTC GAC AAC ACC GGA CCG GTA 225

TTG CGC TGC GGC GCG GCC CCA ACA ATG AGC GCG CCG TGG CGC CGG 270

TGG ATC GAC AGC CTC GTC CCT GGT CCG ATG TCG GGT GGC TGC GGC 315

ACA GCG GTT CAC CTC GGC GAG CCG GTT ATT TCC TAT GAC GTG GCC 360

GAT GAC CCG AAA TTC CGC GGC CCC TTC CGC GCC GCA GCC CTC CAC 405

GAG GGC ATA CGT GCC TGC TGG TCC ACC CCC GTC ACA AGC GGA GAC 450

GGC ACG ATC CTC GGC ACT TTC GCG ATC TAC GGA TCC GTG CCG GCG 495

TTC CCC GCA CAA CAG GAC GTT GCC CTG GTC ACC CAA TGC ACC GAC 540

CTG ACC GCT GCC GTC ATC ACC ACC CAC AAA CTT CAT CAA GAT CTG 585

AGC ATG AGC GAG GAG CGG TTC CGA CGC GCC TTC GAT TCC AAT GTC 630

GTC GGC ATG GCA CTT CTC GAC GAA TCC GGC TCC AGC ATC CGC GTC 675

AAC GAC ACC CTG TGC GCG TTG ACC GCA GCT CCG CCA CGG CGC CTC 720

CTC GGC CAC CCC ATG CAG GAG ATA CTC ACC GCC GAC TCC CGG GAA 765

CCG TTC GCC AAT CAG TTG TCC TCC ATC CGT GAG GGA TTG ACC GAC 810

GGC GGA CAG CTC GAC GGA CGA ATC CAA ACC ACC GGA GGT CGG TGG 855

ATT CCG GTG CAC CTG TCC ATC AGC GGT ATG TGG ACC ACG GAG CGG 900

GAG TTC ATG GGA TTC AGC GTC CAT GTC CTG GAC ATC TCC GAG CGC 945

CTG GCC GCC GAA CGC GCC CGC GAG GAA CAA CTC GAG GCC GAG GTT 990

GCC CGC CAT ACC GCG GAG GAA GCC AGT CGC GCC AAG TCC ACG TTC 1035

CTG TCC GGC ATG ACG CAC GAG GTC CAA ACG CCC ATG GCC GTT ATC 1080

GTC GGA TTC AGT GAG CTA CTC GAG ACG CTG GAC CTG GAT GAA GAA 1125

CGT CGT CAG TGC GCC TAC CGC AAG ATC GGC GAA GCC GCG AAA CAC 1170

GTG ATC TCC CTG GTC GAC GAC GTT CTC GAT ATA GCC AAG ATC GAA 1215

GCC GGC GCT ATC ACT CTG CAG GAC GAA GAC ATC GAC CTG TCC GAA 1260

GAA GTT GCC ACC ATC GTG GAG ATG CTC GAG CCC ATC GCC CGT GAC 1305

CGT GAC CGT GAC GTC TGC CTG CGG TAC GTC CCG CCG CAG ACA CCG 1350

GTG CAC GTG TGC TCG GAC CGG CGG CGG GTG CGG GAA GTG CTG CTC 1395

AAC ATC GTC TCC AAC GGG ATC AAG TAC AAT CGG CTC GGT GGT GTC 1440

GTC GAC CCC CCA ACA GGA TCA GGG GCT GCT CGT CCG CGT CAG ACG 1485

AGG GCC CCG GAC TAC CCA GCG ACG CCG ACG ACG AAC TCT TCG AGC 1530

CCT TCA ACC GGC TGG GAG TCG AGG CCA CGG GGG TGC AAG GGT CGG 1575

GGC TCG GTC TTG CGC TCT CCC GCG CGC TGA 1605

SEQ ID No: 5
LENGTH: 2336
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
ORIGINAL SOURCE
ORGANISM: Rhodococcus erythropolis
STRAIN: SK92
SEQUENCE:

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AACTCGAATC GGAGAATTTC GATGTTGTCG CTGCGGCAGA CGGAGATGCG 150

GCACTGCGTC AGGTCACTGA GAGCGCACCC GATTTGATGG TGCTCGATCT 200

GTCGCTCCCG GGGAAAGGTG GGTTGGAAGT GCTCGCTACG GTCCGCAGAA 250

CCGATGCACT GCCTATCGTC GTGCTCACAG CACGCCGCGA TGAAACCGAA 300

CGGATCGTCG CGCTGGATCT CGGCGCCGAT GACTACGTCA TCAAACCGTT 350

CTCCCCGCGG GAATTGGCCG CCCGTATCCG GGCAGTGCTT CGTCGAACCA 400

CAGCTGAACC CCCACACGAG GCGGCGGTTC AGCGATTCGG TGACCTAGAG 450

ATCGACACCG CTGCCGCGGA GGTTCGGCTC CACGGGATAC CGCTCGAGTT 500

CACCACCAAG GAGTTCGATC TGCTGGCCTA TATGGCCGCA TCACCGATGC 550

AGGTCTTCAG CCGACGCAGA TTGTTGCTCG AGGTGTGGCG ATCGTCGCCC 600

GACTGGCAGC AGGACGCCAC CGTGACCGAG CACGTGCACC GCATTCGCCG 650

CAAGATCGAA GAAGATCCCA CCAAACCGAC GATCCTGCAG ACAGTGCGGG 700

GAGCCGGTTA CCGTTTCGAC GGAGAGCGTG CATGATGACC GACACACTGC 750

CCTCCTCGTC CCGTTGGACC CTTGAAGGCC CGCATCTCCA GCCGCTGCAG 800

GGTGAGGCCC TGGCGGATCT CCACGCCCGT ACGCTCGAGA TGATCACTTC 850

CGGGAGAGAA TTGCACGAGA CACTCGAGGT GGTCGCCCGC GGCATCGAGG 900

AACTGATGCC GGGCAAACGT TGCGCAATTC TGTTGCTCGA CAACACCGGA 950

CCGGTATTGC GCTGCGGCGC GGCCCCAACA ATGAGCGCGC CGTGGCGCCG 1000

GTGGATCGAC AGCCTCGTCC CTGGTCCGAT GTCGGGTGGC TGCGGCACAG 1050

CGGTTCACCT CGGCGAGCCG GTTATTTCCT ATGACGTGGC CGATGACCCG 1100

AAATTCCGCG GCCCCTTCCG CGCCGCAGCC CTCCACGAGG GCATACGTGC 1150

CTGCTGGTCC ACCCCCGTCA CAAGCGGAGA CGGCACGATC CTCGGCACTT 1200

TCGCGATCTA CGGATCCGTG CCGGCGTTCC CCGCACAACA GGACGTTGCC 1250

CTGGTCACCC AATGCACCGA CCTGACCGCT GCCGTCATCA CCACCCACAA 1300

ACTTCATCAA GATCTGAGCA TGAGCGAGGA GCGGTTCCGA CGCGCCTTCG 1350

ATTCCAATGT CGTCGGCATG GCACTTCTCG ACGAATCCGG CTCCAGCATC 1400

CGCGTCAACG ACACCCTGTG CGCGTTGACC GCAGCTCCGC CACGGCGCCT 1450

CCTCGGCCAC CCCATGCAGG AGATACTCAC CGCCGACTCC CGGGAACCGT 1500

TCGCCAATCA GTTGTCCTCC ATCCGTGAGG GATTGACCGA CGGCGGACAG 1550

CTCGACGGAC GAATCCAAAC CACCGGAGGT CGGTGGATTC CGGTGCACCT 1600

GTCCATCAGC GGTATGTGGA CCACGGAGCG GGAGTTCATG GGATTCAGCG 1650

TCCATGTCCT GGACATCTCC GAGCGCCTGG CCGCCGAACG CGCCCGCGAG 1700

GAACAACTCG AGGCCGAGGT TGCCCGCCAT ACCGCGGAGG AAGCCAGTCG 1750

CGCCAAGTCC ACGTTCCTGT CCGGCATGAC GCACGAGGTC CAAACGCCCA 1800

TGGCCGTTAT CGTCGGATTC AGTGAGCTAC TCGAGACGCT GGACCTGGAT 1850

GAAGAACGTC GTCAGTGCGC CTACCGCAAG ATCGGCGAAG CCGCGAAACA 1900

CGTGATCTCC CTGGTCGACG ACGTTCTCGA TATAGCCAAG ATCGAAGCCG 1950

GCGCTATCAC TCTGCAGGAC GAAGACATCG ACCTGTCCGA AGAAGTTGCC 2000

ACCATCGTGG AGATGCTCGA GCCCATCGCC CGTGACCGTG ACCGTGACGT 2050

CTGCCTGCGG TACGTCCCGC CGCAGACACC GGTGCACGTG TGCTCGGACC 2100

GGCGGCGGGT GCGGGAAGTG CTGCTCAACA TCGTCTCCAA CGGGATCAAG 2150

TACAATCGGC TCGGTGGTGT CGTCGACCCC CCAACAGGAT CAGGGGCTGC 2200

TCGTCCGCGT CAGACGAGGG CCCCGGACTA CCCAGCGACG CCGACGACGA 2250

ACTCTTCGAG CCCTTCAACC GGCTGGGAGT CGAGGCCACG GGGGTGCAAG 2300

GGTCGGGGCT CGGTCTTGCG CTCTCCCGCG CGCTGA 2336

Claims

1. A two component regulatory factor which activates a nitrilase gene promoter, comprising a polypeptide having the amino acid sequence of SEQ ID No: 1 and a polypeptide having the amino acid sequence of SEQ ID No: 2.

2. A regulatory factor according to claim 1 whose activation of the nitrilase gene promoter is enhanced in the presence of a nitrile.

3. A DNA molecule encoding a regulatory factor of claim 1 or 2.

4. A DNA molecule according to claim 3 which possesses the nucleotide sequences of SEQ ID Nos: 3 and 4.

5. A recombinant plasmid containing DNA coding for a regulatory factor of claim 1 or 2, a nitrilase gene containing a promoter region and a DNA region capable of replicating in cells of a microorganism belonging to the genus Rhodococcus.

6. A recombinant plasmid according to claim 5 wherein the DNA region capable of replicating in cells of a microorganism belonging to the genus Rhodococcus is from plasmid pRC001 (ATCC 4276), pRC002 (ATCC 14349), pRC003 (ATCC 14348) or pRC004 (IFO 3338).

7. A microorganism belonging to the genus Rhodococcus transformed with a recombinant plasmid of claim 5 or 6.

8. A process for producing nitrilase, which process comprises:

   (i) culturing a microorganism of the genus Rhodococcus containing a DNA molecule encoding the regulatory factor of claim 1 or 2 and a nitrilase gene including its promoter under conditions such that the regulatory factor activates expression of the nitrilase gene; and
   (ii) recovering nitrilase from the culture.

FIG. 1

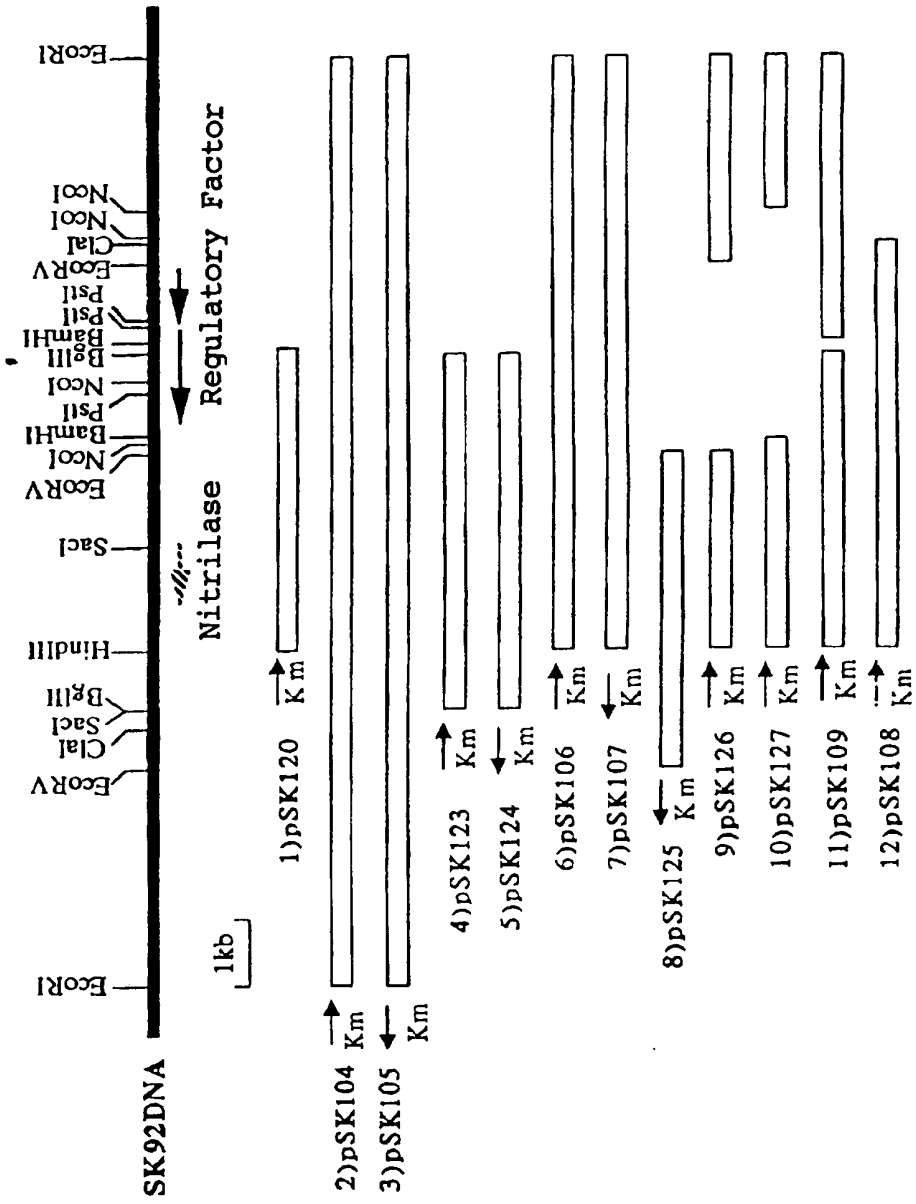
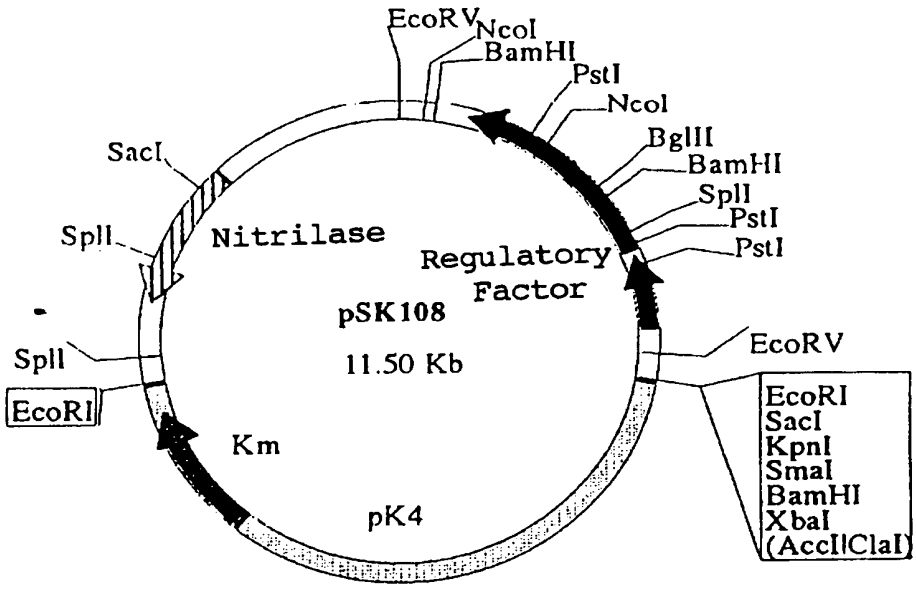

SK92DNA
EcoRI
EcoRV
ClaI
SacI
BglII
HindIII
SacI
EcoRV
NcoI
BamHI
PstI
NcoI
BglII
BamHI
PstI
PstI
EcoRV
ClaI
NcoI
NcoI
EcoRI
1kb
Nitrilase Regulatory Factor
1)pSK120
2)pSK104
3)pSK105
4)pSK123
5)pSK124
6)pSK106
7)pSK107
8)pSK125
9)pSK126
10)pSK127
11)pSK109
12)pSK108
Km

FIG. 2


EcoRV
NcoI
BamHI
PstI
NcoI
BglII
BamHI
SplI
PstI
PstI
EcoRV
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
(AccIIClaI)
SacI
SplI
SplI
EcoRI
Nitrilase
Regulatory
Factor
pSK108
11.50 Kb
Km
pK4